AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

FOOD MICROBIOLOGY

Differential Attachment of *Salmonella enterica* and Enterohemorrhagic *Escherichia coli* to Alfalfa, Fenugreek, Lettuce, and Tomato Seeds

Yue Cui,[a] Ronald Walcott,[b] Jinru Chen[a]

Department of Food Science and Technology, The University of Georgia, Griffin, Georgia, USA[a]; Department of Plant Pathology, The University of Georgia, Athens, Georgia, USA[b]

ABSTRACT Vegetable seeds have the potential to disseminate and transmit foodborne bacterial pathogens. This study was undertaken to assess the abilities of selected *Salmonella* and enterohemorrhagic *Escherichia coli* (EHEC) strains to attach to fungicide-treated versus untreated, and intact versus mechanically damaged, seeds of alfalfa, fenugreek, lettuce, and tomato. Surface-sanitized seeds (2 g) were exposed to four individual strains of *Salmonella* or EHEC at 20°C for 5 h. Contaminated seeds were rinsed twice, each with 10 ml of sterilized water, before being soaked overnight in 5 ml of phosphate-buffered saline at 4°C. The seeds were then vortexed vigorously for 1 min, and pathogen populations in seed rinse water and soaking buffer were determined using a standard plate count assay. In general, the *Salmonella* cells had higher attachment ratios than the EHEC cells. Lettuce seeds by unit weight had the highest numbers of attached *Salmonella* or EHEC cells, followed by tomato, alfalfa, and fenugreek seeds. In contrast, individual fenugreek seeds had more attached pathogen cells, followed by lettuce, alfalfa, and tomato seeds. Significantly more *Salmonella* and EHEC cells attached to mechanically damaged seeds than to intact seeds ($P < 0.05$). Although, on average, significantly more *Salmonella* and EHEC cells were recovered from untreated than fungicide-treated seeds ($P < 0.05$), fungicide treatment did not significantly affect the attachment of individual bacterial strains to vegetable seeds ($P > 0.05$), with a few exceptions. This study fills gaps in the current body of literature and helps explain bacterial interactions with vegetable seeds with differing surface characteristics.

IMPORTANCE Vegetable seeds, specifically sprout seeds, have the potential to disseminate and transmit foodborne bacterial pathogens. This study investigated the interaction between two important bacterial pathogens, i.e., *Salmonella* and EHEC, and vegetable seeds with differing surface characteristics. This research helps understand whether seed surface structure, integrity, and fungicide treatment affect the interaction between bacterial cells and vegetable seeds.

KEYWORDS alfalfa, attachment, EHEC, fenugreek, lettuce, *Salmonella*, tomato, vegetable seeds

Received 17 November 2016 **Accepted** 14 January 2017

Accepted manuscript posted online 27 January 2017

Citation Cui Y, Walcott R, Chen J. 2017. Differential attachment of *Salmonella enterica* and enterohemorrhagic *Escherichia coli* to alfalfa, fenugreek, lettuce, and tomato seeds. Appl Environ Microbiol 83:e03170-16. https://doi.org/10.1128/AEM.03170-16.

Editor Donald W. Schaffner, Rutgers, The State University of New Jersey

Address correspondence to Jinru Chen, jchen@uga.edu.

Globally, fresh produce consumption has increased significantly in the last few decades because of the accrued health benefits (1). Since most fresh produce receives minimal processing and is often eaten raw, it can be a vehicle for transmitting foodborne pathogens (2). It is reported that in the United States, fresh produce was the most common vehicle for transmitting foodborne illness and 19% of the foodborne outbreaks and 24% of the illnesses that took place from 2004 to 2013 were associated with fresh produce (3). In general, enterohemorrhagic *Escherichia coli* (EHEC) and

Salmonella enterica are the major bacterial causes of foodborne illnesses (4). Fresh produce that has been linked to outbreaks of *Salmonella* and EHEC infections includes lettuce and tomato, as well as alfalfa and fenugreek sprouts (5–8).

Fresh produce contamination by pathogenic bacteria such as *Salmonella* and EHEC may occur at pre- or postharvest stages (9). Vegetable seeds can be a potential source and efficient vector of human and plant pathogens. Unsanitized vegetable seeds could lead to the contamination of fresh produce, especially sprouts (10). According to the U.S. Food and Drug Administration (11), most sprout outbreaks have been caused by seeds contaminated with bacterial pathogens before the sprouting process begins. Many pathogens can survive for months under the dry conditions used for seed storage. However, pathogen populations in the seeds are low and unevenly distributed, making them difficult to detect by routine seed testing. Furthermore, seed sanitation treatments have been shown to be ineffective in eliminating bacterial pathogens, especially those that are located in surface cavities (12) or internal vegetable seed tissues (13).

Several studies have investigated the behavior of *Salmonella* and EHEC on various vegetable tissues, such as stems and leaves (14, 15). However, only a few have assessed the attachment ability of bacterial pathogens on vegetable seeds (16–18), particularly lettuce, tomato, and fenugreek seeds. Furthermore, most of the earlier studies concerning bacterial pathogens and vegetable seeds have focused on the efficacy of chemical treatments in reducing seedborne bacterial pathogen populations (19). Physical mechanisms of pathogen attachment to vegetable seeds have not been adequately addressed.

Numerous factors may affect bacterial interactions with seed surfaces. Seed coat characteristics have a significant impact on the levels of contamination by artificially inoculated bacterial pathogens (20). Barak et al. (14) observed fundamental differences between *Salmonella* and *E. coli* O157:H7 in the manner and degree of attachment to alfalfa sprouts. However, this differential attachment behavior has not been reported for alfalfa seeds or other types of vegetable or sprout seeds. This study was undertaken to investigate differences in the abilities of two human enteric pathogens (*Salmonella enterica* and EHEC) to attach to seeds of four different types of vegetables (alfalfa, fenugreek, tomato, and lettuce) and with different surface characteristics (fungicide-treated versus untreated and intact versus mechanically damaged).

RESULTS

Attachment ratios of *Salmonella* and EHEC from different vegetable seeds. Overall, the four *Salmonella* strains used in this study had similar abilities to attach to vegetable seeds (Table 1). However, among the EHEC strains, strain K4499 had a significantly higher ($P < 0.05$) attachment ratio (12.5%) (i.e., the number of attached cells relative to the number of inoculated cells) than the other three EHEC strains. The attachment ratios of H1730 (1.5%) and ATCC BAA-2326 (0.2%) were similar, but they were significantly lower than those of K4499 and F4546 (5.2%). The mean EHEC attachment ratio from the 2-log CFU/ml inoculation level (5.8%) was significantly higher ($P < 0.05$) than that from the 4-log CFU/ml inoculation level (3.9%), but *Salmonella* attachment ratios were similar from the two inoculation levels (Table 1). Lettuce seeds by unit weight (2 g) had the highest numbers of attached *Salmonella* cells (18.7%), followed by tomato (13.2%), alfalfa (11.3%), and fenugreek (6.0%) seeds (Table 1). A similar trend was observed with EHEC cells, except that there was no significant difference in the numbers of cells attached to alfalfa and fenugreek seeds. However, individual fenugreek seeds had the highest numbers of attached *Salmonella* and EHEC cells, followed by lettuce, alfalfa, and tomato seeds (Table 1). With regard to seed surface characteristics, significantly more *Salmonella* and EHEC cells attached to mechanically damaged seeds than to intact seeds (Table 1; $P < 0.05$). *Salmonella* attachment to untreated seeds was significantly higher ($P < 0.05$) than to thiram (dimethylcarbamothioylsulfanyl *N*,*N*-dimethylcarbamodithioate)-treated seeds. In

TABLE 1 Attachment ratios of *Salmonella* and EHEC on alfalfa, fenugreek, lettuce, and tomato seeds

Comparative category	Attachment ratio[a]	
	Salmonella	EHEC
Strains		
S. Cubana/K4499	0.130 A	0.125 A
S. Stanley/F4546	0.124 A	0.052 B
S. Montevideo/H1730	0.123 A	0.015 C
S. Baildon/ATCC BAA-2326	0.116 A	0.002 C
Inoculum levels		
2 log CFU/ml	0.121 A	0.058 A
4 log CFU/ml	0.125 A	0.039 B
Integrity properties		
Damaged	0.174 A	0.064 A
Intact	0.072 B	0.033 B
Treatment practices		
Untreated	0.134 A	0.053 A
Thiram treated	0.112 B	0.044 A
Seed types by unit wt (no. of cells [CFU] attached to individual seed)		
Alfalfa	0.113 C (7.4 C)	0.025C (0.6 C)
Fenugreek	0.060 D (16.6 A)	0.018C (2.5 A)
Lettuce	0.187 A (10.7 B)	0.104A (1.9 B)
Tomato	0.132 B (4.0 D)	0.048B (0.8 C)

[a]Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4. Values within each comparative category (bacterial strain, inoculum level, etc.) in the same column followed by the same letter are not significantly different ($P > 0.05$).

contrast, the numbers of EHEC cells attached to thiram-treated and untreated seeds were not significantly different (Table 1).

Differences in attachment between *Salmonella* and EHEC. In general, the *Salmonella* strains used in this study displayed greater attachment ratios than the EHEC strains (Fig. 1). However, the attachment ratio of K4499 was comparable to those of the four *Salmonella* strains. For most of the bacterial strains included in the study, the numbers of cells attached to vegetable seeds were higher than those that were recovered from seed rinsing waters, except for *Salmonella enterica* serovar Stanley and *E. coli* ATCC BAA-2326.

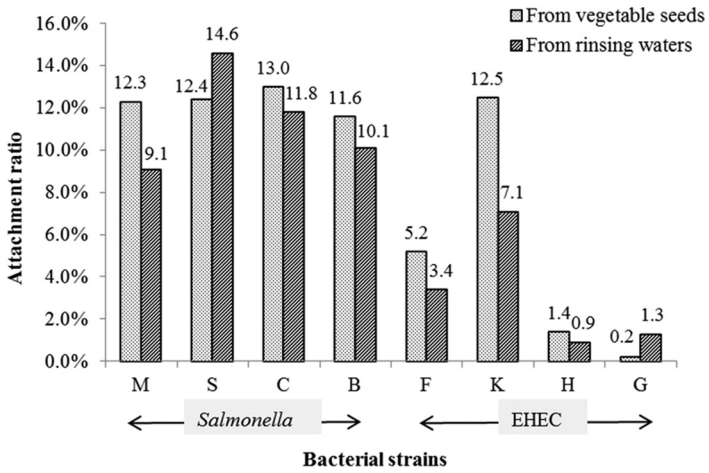

FIG 1 Attachment ratios of *S. enterica* serovars Montevideo (M), Stanley (S), Cubana (C), and Baildon (B), *E. coli* O157:H7 strains F4546 (F), K4499 (K), and H1730 (H), and *E. coli* O104:H4 strain ATCC BAA-2326 (G) to vegetable seeds.

TABLE 2 Attachment of *Salmonella* strains to alfalfa, fenugreek, lettuce, and tomato seeds

	Attachment ratio of indicated *Salmonella* strain from[a]:							
	BSA				NA-TSA			
Seed type	Baildon	Cubana	Montevideo	Stanley	Baildon	Cubana	Montevideo	Stanley
Alfalfa	0.144 Aa	0.177 Aa	0.058 Bb	0.074 Bb	0.159 Aa	0.192 Aa	0.065 Bb	0.091 Bb
Fenugreek	0.062 Ba	0.079 Ba	0.068 Ba	0.029 Bb	0.068 Bb	0.100 Ba	0.063 Bb	0.032 Bc
Lettuce	0.160 Aa	0.199 Aa	0.195 Aa	0.193 Aa	0.169 Ac	0.222 Aab	0.177 Abc	0.242 Aa
Tomato	0.096 Bb	0.065 Bb	0.170 Aa	0.199 Aa	0.107 Bbc	0.073 Bc	0.166 Ab	0.245 Aa

[a]BSA, bismuth sulfite agar; NA-TSA, tryptic soy agar amended with 50 μg/ml nalidixic acid. Values within the same column followed by the same uppercase letter are not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter are not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4.

For EHEC at both inoculum levels and *Salmonella* at the 2-log CFU/ml inoculation level, bacterial populations recovered from each type of seed were similar to the original inoculum levels, except for tomato seeds (see Table S1 in the supplemental material). At the 4-log CFU/ml inoculation level, the number of inoculated *Salmonella* cells was also similar to those recovered from lettuce seeds, and the latter was not significantly different from the numbers of *Salmonella* cells recovered from alfalfa and fenugreek seeds ($P > 0.05$). For tomato seeds, the recovered pathogen levels were significantly lower than the original inoculum levels (Table S1). These data suggest that there was no significant bacterial growth during the 5-h attachment process at 20°C.

Although there were no significant differences in the mean attachment abilities of the four *Salmonella* strains used in this study, individual strains appeared to have a unique affinity to a specific type of vegetable seed (Table 2). Significantly more *S.* Baildon (14.4%) and *S.* Cubana (17.7%) cells attached to alfalfa seeds than cells of *S.* Montevideo (5.8%) and *S.* Stanley (7.4%). Additionally, significantly more *S.* Montevideo (17.0%) and *S.* Stanley (19.9%) cells attached to tomato seeds than cells of the other two *Salmonella* strains (9.6% for *S.* Baildon and 6.5% for *S.* Cubana). *Salmonella* Stanley displayed a relatively low attachment ability to fenugreek seeds (2.9%) in comparison to those of other *Salmonella* serotypes (from 6.2% to 7.9%). Among the EHEC strains, strain K4499 had the highest attachment ratio on every vegetable seed type tested, followed by strain F4546. The attachment ratios of H1730 and ATCC BAA-2326 were significantly lower than those of the other two EHEC strains (Table 3). More F4546 cells attached to lettuce (8.9%) and tomato (7.1%) seeds than alfalfa (3.1%) and fenugreek (1.9%) seeds, while more K4499 cells attached to lettuce seeds (28.7%), followed by tomato (11.0%) and alfalfa (6.0%) seeds. The numbers of K4499 cells recovered from alfalfa seeds were not significantly different from those that attached to fenugreek seeds (4.3%). Significantly more H1730 cells attached to lettuce seeds (3.7%) than to the other three vegetable seed types. Similar numbers of ATCC BAA-2326 cells attached to tomato (0.2%), fenugreek (0.3%), and lettuce (0.4%) seeds, but the number of cells that attached to lettuce seeds was significantly higher than that recovered from alfalfa seeds (0.1%) (Table 3).

TABLE 3 Attachment of EHEC strains to alfalfa, fenugreek, lettuce, and tomato seeds

	Attachment ratio of indicated EHEC strain from[a]:							
	NA-SMAC				NA-TSA			
Seed type	F4546	K4499	H1730	ATCC BAA-2326	F4546	K4499	H1730	ATCC BAA-2326
Alfalfa	0.031 Bb	0.060 BCa	0.008 Bc	0.001 Bc	0.036 Bb	0.079 Ca	0.008 Cc	0.005 Bc
Fenugreek	0.019 Bb	0.043 Ca	0.006 Bc	0.003 ABc	0.031 Bb	0.049 Ca	0.014 Cc	0.005 Bc
Lettuce	0.089 Ab	0.287 Aa	0.037 Ac	0.004 Ac	0.145 Ab	0.372 Aa	0.075 Ac	0.008 ABd
Tomato	0.071 Ab	0.110 Ba	0.007 Bc	0.002 ABc	0.129 Aa	0.150 Ba	0.047 Bb	0.010 Ab

[a]NA-SMAC, sorbitol MacConkey agar supplemented with 50 μg/ml nalidixic acid; NA-TSA, tryptic soy agar supplemented with 50 μg/ml nalidixic acid. Values within the same column followed by the same uppercase letter are not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter are not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4.

TABLE 4 Attachment of *Salmonella* strains to mechanically damaged and intact alfalfa, fenugreek, lettuce, and tomato seeds

	Attachment ratio of indicated *Salmonella* strain from[b]:							
	BSA				NA-TSA			
Seed type[a]	Baildon	Cubana	Montevideo	Stanley	Baildon	Cubana	Montevideo	Stanley
DA-A	0.235 Aa	0.287 Aa	0.099 Cb	0.123 Cb	0.257 Ab	0.314 Aa	0.105 Cc	0.150 Cc
IN-A	0.053 CDb	0.067 CDa	0.017 Dc	0.025 Dc	0.060 DEa	0.070 Ca	0.026 Eb	0.031 Db
DA-Fe	0.083 Cab	0.111 BCa	0.070 Cb	0.034 Dc	0.087 Db	0.151 Ba	0.060 DEbc	0.031 Dc
IN-Fe	0.042 Dab	0.047 DEab	0.067 Ca	0.023 Db	0.049 EFab	0.050 CDab	0.067 CDa	0.033 Db
DA-L	0.155 Bbc	0.140 Bc	0.191 Bab	0.226 Ba	0.162 Cb	0.162 Bb	0.165 Bb	0.264 Ba
IN-L	0.166 Bb	0.258 Aa	0.199 Bab	0.161 Bb	0.175 BCb	0.283 Aa	0.190 Bb	0.220 Bab
DA-T	0.191 Bb	0.130 Bc	0.332 Aa	0.382 Aa	0.202 Bc	0.141 Bd	0.293 Ab	0.434 Aa
IN-T	0.001 Eb	0.001 Eb	0.008 Dab	0.015 Da	0.013 Fb	0.005 Db	0.039 EDab	0.057 Da

[a]DA, damaged; IN, intact; L, lettuce seed; T, tomato seed; A, alfalfa seed; Fe, fenugreek seed.

[b]BSA, bismuth sulfite agar; NA-TSA, tryptic soy agar supplemented with 50 μg/ml nalidixic acid. Values within the same column followed by the same uppercase letter are not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter are not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4. Underlining indicates significantly higher counts on damaged seeds than on intact seeds.

Effect of vegetable seed surface characteristics on pathogen attachment. The numbers of *Salmonella* cells recovered from damaged alfalfa and tomato seeds and *S.* Baildon and *S.* Cubana cells recovered from damaged fenugreek seeds were significantly higher ($P < 0.05$) than those from the corresponding seeds without mechanical damage (Table 4). Furthermore, the numbers of *Salmonella* cells recovered from damaged lettuce seeds and *S.* Montevideo and *S.* Stanley cells recovered from damaged fenugreek seeds were similar to those from their undamaged counterparts, except for lettuce seeds inoculated with *S.* Cubana (Table 4).

The numbers of EHEC cells recovered from damaged tomato seeds, of F4546, K4499, and H1730 cells recovered from damaged alfalfa seeds, of F4546, H1730, and ATCC BAA-2326 cells from damaged fenugreek seeds, and of ATCC BAA-2325 cells from damaged lettuce seeds were significantly higher ($P < 0.05$) than those from their corresponding intact seeds. For the rest of the samples, the numbers of EHEC cells on damaged and intact seeds were statistically similar, except for lettuce seeds that were inoculated with strain K4499 and plated on tryptic soy agar amended with 50 μg/ml nalidixic acid (NA-TSA) (Table 5). Damaged tomato seeds had the lowest number of attached bacterial pathogen cells, except for the samples inoculated with ATCC BAA-2326 (Tables 4 and 5).

Although, on average, significantly more *Salmonella* cells were recovered from untreated seeds than from thiram-treated seeds (Table 1), thiram treatment did not affect the attachment of individual bacterial strains to vegetable seeds, except for lettuce seeds inoculated with *S.* Cubana or *S.* Baildon (Table 6). Similarly, no significant

TABLE 5 Attachment of EHEC strains to mechanically damaged and intact alfalfa, fenugreek, lettuce, and tomato seeds

	Attachment ratio of indicated EHEC strain from[b]:							
	NA-SMAC				NA-TSA			
Seed type[a]	F4546	K4499	H1730	ATCC BAA-2326	F4546	K4499	H1730	ATCC BAA-2326
DA-A	0.049 CDb	0.096 Ca	0.016 Bc	0.001 Cc	0.056 Cb	0.126 Ca	0.012 Bc	0.006 Bc
IN-A	0.013 Eb	0.024 Da	0.001 Cc	0.000 Cc	0.016 CDb	0.031 Da	0.003 Cc	0.003 Bc
DA-Fe	0.031 Dab	0.056 CDa	0.009 Bc	0.005 ABc	0.050 Ca	0.052 CDa	0.022 Bb	0.007 Ab
IN-Fe	0.007 Eb	0.031 CDa	0.002 Cb	0.001 Cb	0.011 Db	0.046 Da	0.006 Cb	0.004 Bb
DA-L	0.099 Bb	0.252 ABa	0.033 Abc	0.008 Ac	0.138 Bb	0.354 Ba	0.065 Abc	0.013 Ac
IN-L	0.079 BCb	0.323 Aa	0.040 Abc	0.001 Cc	0.151 Bb	0.390 Aa	0.086 Ac	0.002 Bd
DA-T	0.133 Ab	0.217 Ba	0.014 Bc	0.003 Bc	0.227 Ab	0.281 Ba	0.094 Ac	0.017 Ad
IN-T	0.009 Ea	0.004 Dab	0.001 Cb	0.001 Cb	0.031 CDa	0.019 Dab	0.001 Cb	0.003 Bb

[a]DA, damaged; IN, intact; L, lettuce seed; T, tomato seed; A, alfalfa seed; Fe, fenugreek seed.

[b]NA-SMAC, sorbitol MacConkey agar amended with 50 μg/ml nalidixic acid; NA-TSA, tryptic soy agar amended with 50 μg/ml nalidixic acid. Values within the same column followed by the same uppercase letter are not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter are not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4. Underlining indicates significantly higher counts on damaged seeds than intact seeds.

TABLE 6 Attachment of *Salmonella* strains to thiram-treated and untreated alfalfa, fenugreek, lettuce, and tomato seeds

	Attachment ratio of indicated *Salmonella* strain from[b]:							
	BSA				NA-TSA			
Seed type[a]	Baildon	Cubana	Montevideo	Stanley	Baildon	Cubana	Montevideo	Stanley
TR-A	0.136 ABa	0.150 BCa	0.059 Bb	0.063 Bb	0.152 ABCab	0.180 Ba	0.066 Bc	0.081 Bbc
UN-A	0.152 ABab	0.204 ABa	0.058 Bc	0.085 Bbc	0.165 ABab	0.203 ABa	0.064 Bc	0.100 Bbc
TR-Fe	0.062 Cab	0.089 CDa	0.058 Bb	0.026 Bc	0.073 Db	0.130 BCa	0.068 Bbc	0.031 Bc
UN-Fe	0.063 Cab	0.069 Da	0.079 Ba	0.031 Bb	0.063 Dab	0.070 Ca	0.059 Bab	0.033 Bb
TR-L	0.129 Bb	0.136 BCDab	0.173 Aab	0.181 Aa	0.146 ABCb	0.174 Bab	0.171 Aab	0.207 Aa
UN-L	0.191 Aa	0.261 Aa	0.217 Aa	0.205 Aa	0.191 Abc	0.271 Aab	0.183 Ac	0.277 Aa
TR-T	0.097 BCab	0.066 Db	0.179 Aa	0.188 Aa	0.115 BCDbc	0.088 Cc	0.186 Aab	0.265 Aa
UN-T	0.096 BCb	0.064 Db	0.160 Aab	0.209 Aa	0.100 Db	0.058 Cb	0.146 Aab	0.226 Aa

[a]TR, thiram treated; UN, untreated; L, lettuce seed; T, tomato seed; A, alfalfa seed; Fe, fenugreek seed.

[b]BSA, bismuth sulfite agar; NA-TSA, tryptic soy agar supplemented with 50 μg/ml nalidixic acid. Values within the same column followed by the same uppercase letter are not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter are not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4. Underlining indicates similar counts on thiram-treated and untreated seeds.

difference in attachment ratio of EHEC cells was observed between thiram-treated seeds and untreated seeds, except for lettuce seeds inoculated with strain H1730 (Table 7).

Surface morphology of intact and damaged vegetable seeds. Fenugreek seeds had a cuboid shape and were the largest of the four types of seeds used in this study. Lettuce seeds were long and thin and had granular structures on their surfaces (Fig. 2). Oval-shaped alfalfa seeds had relatively smooth surfaces, while round tomato seeds had a rough surface texture (Fig. 2). The scanning electron micrographs of the seed surfaces revealed exposed cavities on mechanically damaged fenugreek and alfalfa seeds and seed debris on mechanically damaged lettuce and tomato seeds (Fig. 2). More-detailed scanning electron micrographs of seed surface morphology revealed regular nodes and crevices on fenugreek seeds (Fig. 3A), crevices and irregular nodes on lettuce seeds (Fig. 3B), pubescent covering (fuzz) and crevices on tomato seeds (Fig. 3C), and slight cracks on alfalfa seeds (Fig. 3D).

DISCUSSION

***Salmonella* and EHEC attachment to vegetable seeds.** The present study revealed that, on average, *S. enterica* had greater attachment ratios than EHEC. Similar observations were reported in several previous studies involving alfalfa and bean sprouts (14, 21), cantaloupe rind surface (22), and food contact surfaces (23). Difference in cell surface hydrophobicity between *Salmonella* and EHEC cells was believed to be one of the contributing factors for the observed phenomenon (22–25). However, contradictory

TABLE 7 Attachment of EHEC strains to mechanically damaged and intact alfalfa, fenugreek, lettuce, and tomato seeds

	Attachment ratio of indicated EHEC strain from[b]:							
	NA-SMAC				NA-TSA			
Seed type[a]	F4546	K4499	H1730	ATCC BAA-2326	F4546	K4499	H1730	ATCC BAA-2326
TR-A	0.029 DEab	0.045 Ba	0.011 BCbc	0.000 Ac	0.038 Bb	0.076 BCDa	0.008 Dc	0.004 Bc
UN-A	0.032 CDEb	0.075 Ba	0.006 Cb	0.000 Ab	0.034 Bb	0.081 BCDa	0.007 Db	0.005 Bb
TR-Fe	0.017 Eb	0.040 Ba	0.005 Cbc	0.004 Ac	0.034 Ba	0.039 Da	0.013 CDb	0.007 ABb
UN-Fe	0.021 Eb	0.046 Ba	0.006 Cbc	0.002 Ac	0.027 Bb	0.058 CDa	0.015 CDbc	0.004 Bc
TR-L	0.072 ABCb	0.260 Aa	0.025 Bbc	0.003 Ac	0.122 Ab	0.323 Aa	0.046 BCc	0.007 ABc
UN-L	0.106 Ab	0.315 Aa	0.049 Abc	0.005 Ac	0.168 Ab	0.421 Aa	0.105 Abc	0.008 ABc
TR-T	0.063 BCDb	0.113 Ba	0.010 BCc	0.002 Ac	0.131 Aab	0.156 Ba	0.060 Bbc	0.009 ABc
UN-T	0.079 ABa	0.108 Ba	0.005 Cb	0.001 Ab	0.126 Aa	0.144 BCa	0.034 BCDb	0.011 Ab

[a]TR, thiram treated; UN, untreated; L, lettuce seed; T, tomato seed; A, alfalfa seed; Fe, fenugreek seed.

[b]NA-SMAC, sorbitol MacConkey agar amended with 50 μg/ml nalidixic acid; NA-TSA, tryptic soy agar amended with 50 μg/ml nalidixic acid. Values within the same column followed by the same uppercase letter are not significantly different ($P > 0.05$); values within the same row followed by the same lowercase letter are not significantly different ($P > 0.05$). Statistical comparisons were based on Fisher's least significant difference test using SAS statistical software version 9.4. Underlining indicates similar counts on thiram-treated and untreated seeds.

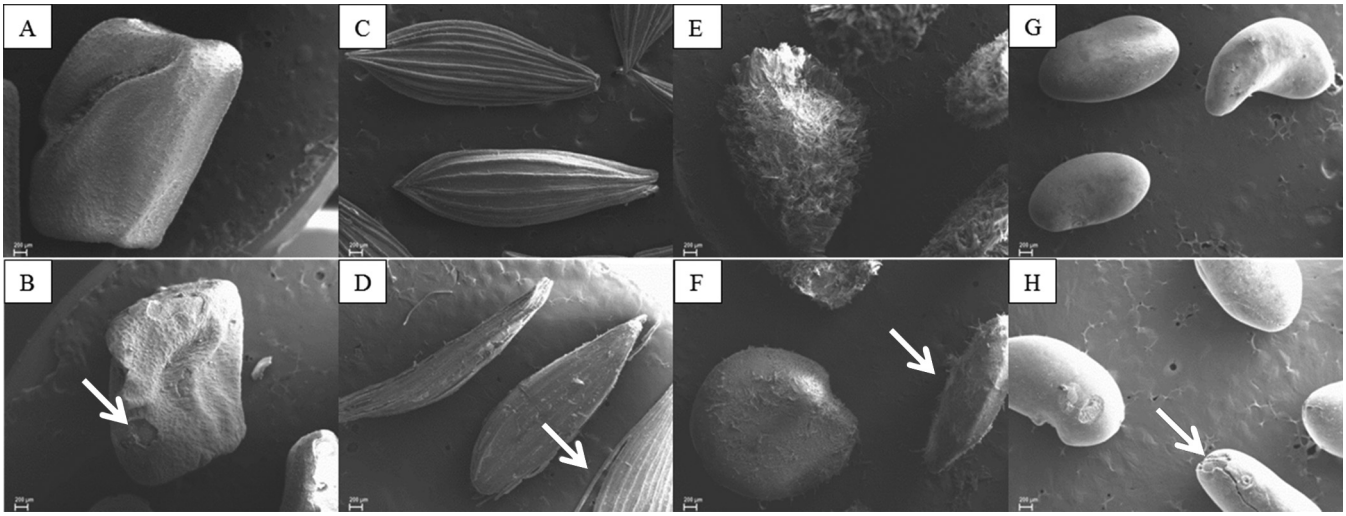

FIG 2 Scanning electron micrographs of selected vegetable seeds with different seed coat integrity properties. (A) Intact fenugreek seed; (B) damaged fenugreek seed; (C) intact lettuce seed; (D) damaged lettuce seed; (E) intact tomato seed; (F) damaged tomato seed; (G) intact alfalfa seed; (H) damaged alfalfa seed. Bars, 200 μm. Arrows indicate mechanical damage on seed surfaces.

findings have also been reported. Takeuchi et al. (15) observed that more *E. coli* O157:H7 cells than *S.* Typhimurium cells attached to lettuce leaf surfaces. It is possible that cell surface hydrophobicity varies among bacterial species/serotypes as well as among individual strains within a bacterial species/serotype. Furthermore, intrinsic cell factors other than cell surface hydrophobicity may play important roles in the interaction between bacterial cells and contact surfaces (26).

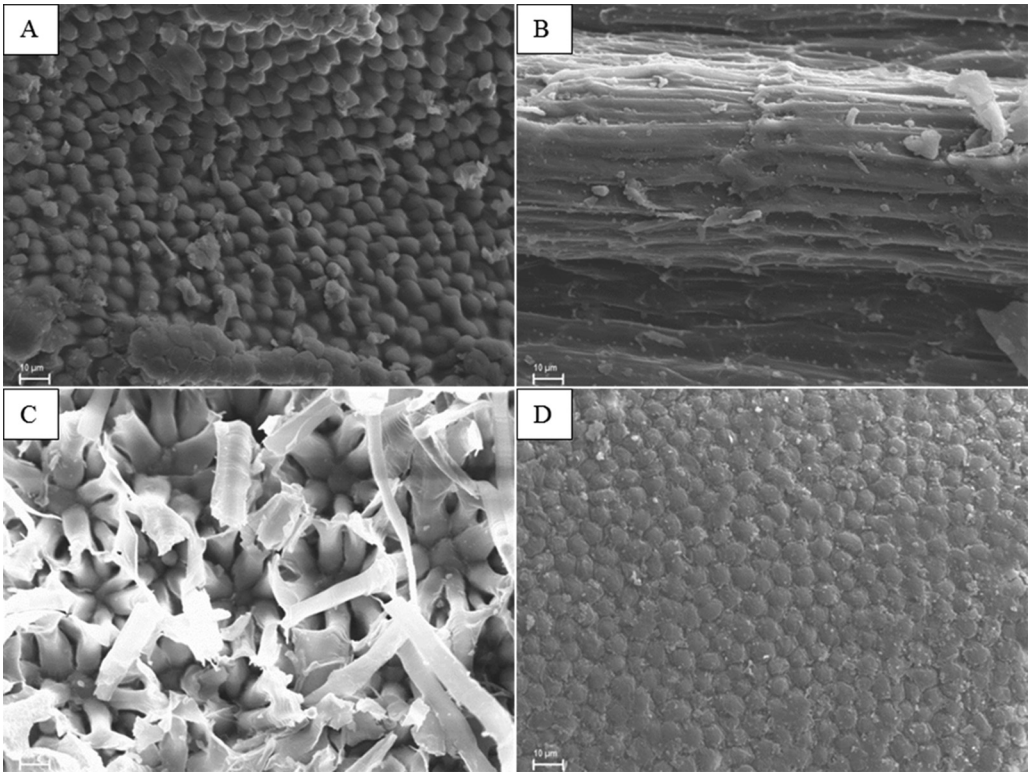

FIG 3 Scanning electron micrographs showing surface morphologies of fenugreek (A), lettuce (B), tomato (C), and alfalfa (D) seeds. Bars, 10 μm.

Among the EHEC strains used in this study, *E. coli* O104:H4 ATCC BAA-2326 had the lowest attachment potential (Fig. 1). This bacterial strain was isolated from a fenugreek sprout-associated outbreak in Germany in 2011 which affected 3,842 people in a dozen countries (7). ATCC BAA-2326 evolved from an enteroaggregative *E. coli* (EAEC) strain that acquired the genes for Shiga toxin production (7). EAEC strains produce a large amount of extracellular polymeric substances (EPS) (27). While EPS enhance cell aggregation and biofilm formation, they usually impair initial bacterial attachment to contact surfaces (28, 29).

The numbers of the bacterial cells attached to vegetable seeds were generally higher than those that were recovered from seed rinsing waters, except for *S.* Stanley and *E. coli* ATCC BAA-2326 (Fig. 1). This suggests that more cells of these two bacteria were loosely associated with seed surfaces and more easily rinsed away. This observation was incongruent with a previous study by Barak et al. (14), who reported that more *Salmonella* and *E. coli* O157: H7 cells were recovered from sprout rinse water than from sprout samples themselves. This inconsistency may be due to specific bacterial strains and experimental conditions used in the two studies. Additionally, seeds and seed sprouts have different surface characteristics. Surfaces of seeds are rougher and have irregular shapes and larger surface areas, while sprouts are thinner, longer, and smoother and have smaller surface areas.

We observed that the attachment ratios of *Salmonella* cells ranged from 2.9% on fenugreek seeds contaminated with *S.* Stanley to 19.9% on lettuce seeds contaminated with *S.* Cubana and tomato seeds contaminated by *S.* Stanley. By comparison, EHEC attachment ratios ranged from 0.1% on alfalfa seeds contaminated with ATCC BAA-2326 to 28.7% on lettuce seeds contaminated with K4499 (Tables 2 and 3). Most of the previous studies reported bacterial pathogen levels on vegetable seeds in log CFU per gram, rather than in terms of attachment ratio. Fransisca et al. (18) exposed 300 g of alfalfa seeds to 300 ml of a 10^7 CFU/ml *E. coli* O157 culture for 2 min, followed by rinsing for 20 min with water, and 3.16 CFU/g of *E. coli* O157 cells were recovered from alfalfa seeds, which was equivalent to an attachment ratio of 0.01%. This value was lower than the attachment ratios observed for alfalfa seeds contaminated with EHEC in the present study (Table 3). Fransisca et al. (18) reported that each gram of seeds was exposed to 10^7 CFU of *E. coli* cells, while in our study each gram of vegetable seeds was exposed to 10^3 to 10^5 CFU of pathogen cells. In addition, different bacterial strains and attachment conditions were used in the two studies. Higher cell concentrations were also used as inocula in several other studies (30–32), and as expected, the attachment ratios calculated from these studies were lower than what was observed in the present study.

In the current study, the inoculation level had no significant effect on *Salmonella* attachment ratios, indicating that the number of *Salmonella* cells attached to seed surfaces increased as inoculum concentration increased. However, EHEC attachment ratios from the 2-log CFU/ml inoculation level were significantly higher than those from the 4-log CFU/ml inoculation level. The precise reason for the observed phenomenon is not clear.

Effect of seed surface properties on bacterial attachment. The surfaces of vegetable seeds are complex, and different types of seeds have different surface properties. As a result, they have different potentials to attract bacterial cells. Lettuce seeds by unit weight had the highest number of *Salmonella* and EHEC cells, followed by tomato, alfalfa, and fenugreek seeds (Table 1). According to scanning electron micrographs, lettuce seed surfaces have nodes and crevices, while tomato seeds are pubescent. These two types of seeds have a rougher surface than alfalfa seeds (Fig. 3), which explains, in part, why pathogen attachment ratios from alfalfa seeds were lower (Table 1). This observation is supported by previous studies that demonstrated that wrinkled or rough seeds were likely to harbor more bacteria and were more resistant to sanitizers than smooth seeds (17, 18). Additionally, in the current study, alfalfa seeds clumped together in an aqueous environment, which might have prevented bacterial

cells from attaching to their surfaces. Among the four seed types used in the present study, fenugreek seeds by unit weight had the lowest *Salmonella* and EHEC attachment ratios. Fenugreek seeds are larger and heavier, and by unit weight they have smaller surface areas to interact with bacterial cells than other seeds. It is worth noting that the fact that the two types of sprout seeds used in this study did not have higher numbers of *Salmonella* and EHEC cells attached does not make them microbiologically safer. The increase in bacterial population during seed germination and sprouting can still pose significant threats to consumer health.

Salmonella and EHEC attachment ratios from intact tomato seeds were the lowest among all the seed types tested. In addition, the total numbers of bacterial pathogens recovered from tomato seeds were significantly lower than the numbers of inoculated bacterial pathogen cells. Commercial tomato seeds are processed through a natural fermentation process to eliminate chemical compounds that inhibit germination (32). Although tomato seeds are washed and dried after fermentation, residual fermentation compounds such as lycopene may be present in seed coat hairs, leading to an acidic seed surface (pH ca. 2.6; detailed data not shown). Furthermore, tomato seed fermentation mixtures may contain antimicrobial compounds that inhibit the attachment and viability of bacterial pathogens. Arkoun et al. (33) reported that lactic acid bacteria isolated from fermented tomato fruits produced a bacteriocin-like substance that had inhibited a variety of Gram-negative bacteria, including *E. coli*.

Seed integrity properties, fungicide treatment, and bacterial attachment. The numbers of bacterial cells that attached to mechanically damaged seeds were significantly higher than those that attached to intact seeds, with a few exceptions. The microtopography of tested seeds revealed that damaging seed coats resulted in cracks that can provide physical protection for bacterial cells. This may have made it more difficult for loosely attached bacterial cells to be rinsed away. This observation is in agreement with previous studies that showed that cells of *L. monocytogenes* and *E. coli* O157:H7 were more likely to attach to cut edges of cabbage and lettuce (15, 26, 34). One explanation for the recovery of higher numbers of bacterial cells from damaged seeds than from intact seeds is that mechanical damage may result in increased uptake of water and increased leakage of solutes from seeds (35), which may promote attachment and growth of bacterial pathogens. However, according to the results shown in Table S1, significant bacterial multiplication did not occur during the attachment experiment.

Although significantly more *Salmonella* and EHEC cells were recovered from untreated seeds than from treated seeds, treatment of vegetable seeds with thiram did not affect the attachment of individual bacterial strains to vegetable seeds. The only exception was lettuce seeds. According to the U.S. Environmental Protection Agency, thiram is a nonsystemic fungicide, seed protectant, and animal repellent. Thiram is applied to seeds prior to planting as a dust, wettable powder, or liquid. The chemical has antibacterial (36) and antifungal (37) activities; however, no significant difference in the levels of bacterial attachment was found between thiram-treated and untreated seeds in the present study. This could be due to the fact that most of the thiram might have been rinsed off during seed sanitization and subsequent rinsing.

In summary, we observed the interactions between two important bacterial pathogens, i.e., *Salmonella* and EHEC, and vegetable seeds with different surface characteristics. The study provides a better understanding of whether seed surface structure, integrity, and fungicide treatment affect bacterial interactions with vegetable seed surfaces. Bacterial pathogen attachment to vegetable seeds is the first step in colonization. The fate of pathogen cells after the initial attachment step also has a significant impact on fresh produce safety. A study is under way in our laboratory to assess whether *Salmonella* and EHEC cells could migrate from contaminated vegetable seeds to different tissues of seed sprouts and vegetable seedlings.

TABLE 8 Bacterial strains used in this study

Bacterial strain	Abbreviation	Description/source	Reference
S. enterica serovar Baildon	B	Tomato-associated outbreak strain	6
S. enterica serovar Cubana	C	Alfalfa sprout-associated outbreak strain	40
S. enterica serovar Stanley	S	Alfalfa sprout-associated outbreak strain	41
S. enterica serovar Montevideo	M	Tomato-associated outbreak strain	42
E. coli O157:H7 F4546	F	Alfalfa sprout-associated outbreak strain	43
E. coli O157:H7 K4499	K	Spinach-associated outbreak strain	
E. coli O157:H7 H1730	H	Lettuce-associated outbreak strain	
E. coli O104:H4 ATCC BAA-2326	G	Fenugreek-associated outbreak strain	7

MATERIALS AND METHODS

Bacterial strains, growth media and vegetable seeds. Four *S. enterica* strains, three *E. coli* O157:H7 strains, and one *E. coli* O104:H4 strain were used in this study (Table 8). The bacterial strains were stored at −70°C and recovered on tryptic soy agar (TSA) at 37°C for 16 h. The resulting cultures were purified on bismuth sulfite agar (BSA; Becton, Dickinson, Sparks, MD), sorbitol MacConkey (SMAC; Becton Dickinson, Sparks, MD) agar, and MacConkey (MAC; Becton, Dickinson, Sparks, MD) agar, respectively. Spontaneous mutant cells resistant to 50 μg/ml nalidixic acid (NA; MP Biomedicals, Santa Ana, CA) were selected and used in the experiments.

Thiram (dimethylcarbamothioylsulfanyl *N,N*-dimethylcarbamodithioate)-treated and untreated fenugreek (*Trigonella foenum-graecum*, cultivar unidentified), lettuce (*Lactuca sativa* cv. Iceberg), and tomato (*Solanum lycopersicum* cv. Roma) seeds, as well as untreated alfalfa (*Medicago sativa*, cultivar unidentified) seeds, were obtained from a commercial source (Otis S. Twilley Seed Co. Inc., Hodges, SC) and stored at 10°C within a month before use. Commercial alfalfa seeds were treated with thiram 75 WP wettable powder fungicide (Chemtura, Pekin, IL) in-house at a rate of 1.8 g/500 g of seeds in accordance with instructions provided by Norac Concepts, Inc. (38). In order to mechanically damage seeds, 50 g of each seed type was blended in a 14-speed blender (Oster, Milwaukee, WI) for 30 s, and seed debris was removed using a sterilized, fine-mesh sieve (Walmart, Bentonville, AR; hole size, 0.05 mm). Thiram-treated intact, untreated intact, thiram-treated mechanically damaged, and untreated mechanically damaged seeds of alfalfa, fenugreek, lettuce, and tomato were used in the study.

Bacterial attachment to seed surfaces. The experiment involving bacterial attachment to seed surfaces was performed based on the method described by Barak et al. (14) and Darsonval et al. (39) with modifications. Two grams of each type of seed (described above) was placed in 50-ml centrifuge tubes (Fisher Scientific, Asheville, NC) and sanitized with 10 ml of a 20,000-ppm sodium hypochlorite solution (pH 6.8; Becton Dickinson, Sparks, MD) at room temperature for 15 min with gentle mixing. The seeds were then neutralized with 10 ml of Dey-Engley neutralizing broth (Becton Dickinson) for 10 min with gentle mixing and rinsed twice, each with 10 ml of sterilized deionized water for 1 min. An overnight culture of each *Salmonella* and EHEC strain grown in Luria-Bertani no-salt broth supplemented with NA (50 μg/ml) was diluted in sterilized water, and 20 ml of two concentrations (10^2 and 10^4 CFU/ml) of each inoculum was added to the centrifuge tubes with sanitized seeds. The precise inoculation levels were determined by plating 0.1 ml of appropriately diluted cell suspensions on TSA amended with NA. Vegetable seeds in the centrifuge tubes were agitated horizontally at 100 rpm in an orbital platform shaker (model 3520; Lab-Line, IL, USA) at 20°C for 5 h. The inocula were then decanted to sterilized test tubes, and seeds were rinsed twice, each with 10 ml of sterilized water for 30 s with gentle mixing. The rinse water from each seed type was collected into a sterilized test tube. Seeds were then soaked overnight at 4°C in 5 ml of phosphate-buffered saline (pH 7.4) to release attached bacterial cells. Each sample in the experiment was duplicated, and all experiments were conducted twice.

Quantification of bacteria. After being soaked at 4°C overnight, seed samples were vortexed at 3,200 rpm (Fisher Scientific, Asheville, NC) for 1 min. The resulting samples were 10-fold serially diluted, and appropriate dilutions of samples inoculated with *Salmonella* were plated on BSA. Those that were inoculated with *E. coli* O157 or O104 were plated on SMAC or MAC agar amended with NA, respectively. Additionally, all samples were plated on TSA amended with NA (NA-TSA). Bacterial populations in seed rinse water were also determined, as described previously. The ratio of the number of attached cells to the number of inoculated cells (attachment ratio) and the ratio of the number of the cells recovered from seed rinse water to the number of inoculated cells were both reported. The total populations of unattached cells in spent inoculum suspension, attached cells recovered from vegetable seeds, and loosely attached cells in seed rinse water were compared with the bacterial counts in the original inocula. A significant difference between the sum population and the cell count in the inocula indicates bacterial growth during each experiment.

Scanning electron microscopy. To observe the surface morphology of dry vegetable seeds used in the study, scanning electron microscopy was performed according to the approach outlined in the user's manual. Each type of dry seed was mounted directly on stubs using double-sided adhesive tape and sputter-coated with gold using an SPI module sputter coater (model 11428-AB; Structure Probe, Inc., West Chester, PA). The surface morphologies of seeds were examined using a Zeiss 1450EP scanning electron microscope (Carl Zeiss, Inc., Thornwood, NY). Digital images (65× [Fig. 2] and 2,000× [Fig. 3]) were captured using SmartSEM (Carl Zeiss, Inc., Thornwood, NY).

Statistical analysis. To estimate differences among attachment ratios of each tested bacterial strain on vegetable seeds, Fisher's least significant difference test in a general linear model was used for separation of means based on a 95% confidence level using SAS (version 9.4; SAS Institute Inc., Cary, NC). The same statistical test was used to analyze the differences in pathogen attachment to seeds with differing integrities and fungicide treatments.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03170-16.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank John Shields for providing technical assistance with scanning electron microscopy, Norac Concepts, Inc., for donating the fungicide thiram, and Larry Beuchat and Mark Harrison for providing some of the bacterial strains.

This material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 2014-67017-21705.

REFERENCES

1. Esposito K, Giugliano D. 2011. Increased consumption of green leafy vegetables, but not fruit, vegetables or fruit and vegetables combined, is associated with reduced incidence of type 2 diabetes. Evid Based Med 16:27–28. https://doi.org/10.1136/ebm1152.
2. Scharff RL. 2012. Economic burden from health losses due to foodborne illness in the United States. J Food Prot 75:123–131. https://doi.org/10.4315/0362-028X.JFP-11-058.
3. Fischer N, Bourne A, Plunkett D. 2015. A review of foodborne illness in the U.S. from 2004-2013. Center for Science in the Public Interest, Washington, DC.
4. Warriner K, Huber A, Namvar A, Fan W, Dunfield K. 2009. Recent advances in the microbial safety of fresh fruits and vegetables. Adv Food Nutr Res 57:155–208. https://doi.org/10.1016/S1043-4526(09)57004-0.
5. Slayton RB, Turabelidze G, Bennett SD, Schwensohn CA, Yaffee AQ, Khan F, Butler C, Trees E, Ayers TL, Davis ML, Laufer AS, Gladbach S, Williams I, Gieraltowski LB. 2013. Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 associated with romaine lettuce consumption, 2011. PLoS One 8:e55300. https://doi.org/10.1371/journal.pone.0055300.
6. Cummings K, Barrett E, Mohle-Boetani JC, Brooks JT, Farrar J, Hunt T, Fiore A, Komatsu K, Werner SB, Slutsker L. 2001. A multistate outbreak of *Salmonella enterica* serotype Baildon associated with domestic raw tomatoes. Emerg Infect Dis 7:1046–1048. https://doi.org/10.3201/eid0706.010625.
7. Beutin L, Martin A. 2012. Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. J Food Prot 75:408–418. https://doi.org/10.4315/0362-028X.JFP-11-452.
8. CDC. 2007. Multistate outbreaks of *Salmonella* infections associated with raw tomatoes eaten in restaurants—United States, 2005-2006. MMWR Morb Mortal Wkly Rep 56:909–911.
9. Olaimat AN, Holley RA. 2012. Factors influencing the microbial safety of fresh produce: a review. Food Microbiol 32:1–19. https://doi.org/10.1016/j.fm.2012.04.016.
10. Hanning IB, Ricke SC, Nutt JD. 2009. Salmonellosis outbreaks in the United States due to fresh produce: sources and potential intervention measures. Foodborne Pathog Dis 6:635–648. https://doi.org/10.1089/fpd.2008.0232.
11. National Advisory Committee on Microbiological Criteria for Foods. 1999. Microbiological safety evaluations and recommendations on sprouted seeds. Int J Food Microbiol 52:123–153. https://doi.org/10.1016/S0168-1605(99)00135-X.
12. Beuchat L. 1997. Comparison of chemical treatments to kill *Salmonella* on alfalfa seeds destined for sprout production. Int J Food Microbiol 34:329–333. https://doi.org/10.1016/S0168-1605(96)01202-0.
13. Fu TJ, Reineke KF, Chirtel S, VanPelt OM. 2008. Factors influencing the growth of *Salmonella* during sprouting of naturally contaminated alfalfa seeds. J Food Prot 71:888–896. https://doi.org/10.4315/0362-028X-71.5.888.
14. Barak JD, Whitehand LC, Charkowski AO. 2002. Differences in attachment of *Salmonella enterica* serovars and *Escherichia coli* O157:H7 to alfalfa sprouts. Appl Environ Microbiol 68:4758–4763. https://doi.org/10.1128/AEM.68.10.4758-4763.2002.
15. Takeuchi K, Matute CM, Hassan AN, Frank JF. 2000. Comparison of the attachment of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Pseudomonas fluorescens* to lettuce leaves. J Food Prot 63:1433–1437. https://doi.org/10.4315/0362-028X-63.10.1433.
16. Van der Linden I, Cottyn B, Uyttendaele M, Vlaemynck G, Maes M, Heyndrickx M. 2013. Long-term survival of *Escherichia coli* O157:H7 and *Salmonella enterica* on butterhead lettuce seeds, and their subsequent survival and growth on the seedlings. Int J Food Microbiol 161:214–219. https://doi.org/10.1016/j.ijfoodmicro.2012.12.015.
17. Charkowski AO, Sarreal CZ, Mandrell RE. 2001. Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. J Food Prot 64:1292–1298. https://doi.org/10.4315/0362-028X-64.9.1292.
18. Fransisca L, Feng H. 2012. Effect of surface roughness on inactivation of *Escherichia coli* O157:H7 87-23 by new organic acid-surfactant combinations on alfalfa, broccoli, and radish seeds. J Food Prot 75:261–269. https://doi.org/10.4315/0362-028X.JFP-11-279.
19. Buchholz A, Matthews KR. 2010. Reduction of *Salmonella* on alfalfa seeds using peroxyacetic acid and a commercial seed washer is as effective as treatment with 20 000 ppm of Ca(OCl)2. Lett Appl Microbiol 51:462–468. https://doi.org/10.1111/j.1472-765X.2010.02929.x.
20. Rajkowski KT. 2009. Percent moisture and seed coat characteristics of alfalfa seeds after artificial inoculation. J Food Safety 29:224–235. https://doi.org/10.1111/j.1745-4565.2009.00152.x.
21. Han R, Klu YA, Chen J. 2014. Attachment and biofilm formation by selected strains of *Salmonella enterica* and *Escherichia coli* of fresh

produce origin. Abstr Int Assoc Food Prot 104th Annu Meet, Indianapolis, IN.
22. Ukuku DO, Fett WF. 2002. Relationship of cell surface charge and hydrophobicity to strength of attachment of bacteria to cantaloupe rind. J Food Prot 65:1093–1099. https://doi.org/10.4315/0362-028X-65.7.1093.
23. Palmer J, Flint S, Brooks J. 2007. Bacterial cell attachment, the beginning of a biofilm. J Ind Microbiol Biotechnol 34:577–588. https://doi.org/10.1007/s10295-007-0234-4.
24. Stenström TA. 1989. Bacterial hydrophobicity, an overall parameter for the measurement of adhesion potential to soil particles. Appl Environ Microbiol 55:142–147.
25. Dickson JS, Koohmaraie M. 1989. Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. Appl Environ Microbiol 55:832–836.
26. Macarisin D, Patel J, Bauchan G, Giron JA, Sharma VK. 2012. Role of curli and cellulose expression in adherence of *Escherichia coli* O157:H7 to spinach leaves. Foodborne Pathog Dis 9:160–167. https://doi.org/10.1089/fpd.2011.1020.
27. Okhuysen PC, Dupont HL. 2010. Enteroaggregative *Escherichia coli* (EAEC): a cause of acute and persistent diarrhea of worldwide importance. J Infect Dis 202:503–505. https://doi.org/10.1086/654895.
28. Petrova OE, Sauer K. 2012. Sticky situations: key components that control bacterial surface attachment. J Bacteriol 194:2413–2425. https://doi.org/10.1128/JB.00003-12.
29. Beloin C, Roux A, Ghigo JM. 2008. *Escherichia coli* biofilms. Curr Top Microbiol Immunol 322:249–289.
30. Bari ML, Enomoto K, Nei D, Kawamoto S. 2010. Practical evaluation of mung bean seed pasteurization method in Japan. J Food Prot 73:752–757. https://doi.org/10.4315/0362-028X-73.4.752.
31. Hong EJ, Kang DH. 2016. Effect of sequential dry heat and hydrogen peroxide treatment on inactivation of Salmonella Typhimurium on alfalfa seeds and seeds germination. Food Microbiol 53:9–14. https://doi.org/10.1016/j.fm.2015.08.002.
32. Nemati H, Nazdar T, Azizi M, Arouiee H. 2010. The effect of seed extraction methods on seed quality of two cultivar's tomato (*Solanum lycopersicum L.*). Pak J Biol Sci 13:814–820. https://doi.org/10.3923/pjbs.2010.814.820.
33. Arkoun FC, Abbas DK, Zighen TK. 2015. Evaluation of lactic acid bacteria isolated from fermented tomatoes to produce antimicrobial activities against several bacteria and fungi. Adv Food Sci Technol 3:332–338.
34. Ells TC, Hansen LT. 2006. Strain and growth temperature influence *Listeria spp*. attachment to intact and cut cabbage. Int J Food Microbiol 111:34–42. https://doi.org/10.1016/j.ijfoodmicro.2006.04.033.
35. Hwang S, Gossen B, Chang K, Turnbull G, Howard R. 2001. Effect of seed damage and metalaxyl seed treatment on pythium seedling blight and seed yield of field pea. Can J Plant Sci 81:509–517. https://doi.org/10.4141/P00-155.
36. Chaube H, Pundhir V. 2005. Crop diseases and their management. PHI Learning Pvt. Ltd., Delhi, India.
37. Xue J, Luo Z, Li P, Ding Y, Cui Y, Wu Q. 2014. A residue-free green synergistic antifungal nanotechnology for pesticide thiram by ZnO nanoparticles. Sci Rep 4:5408. https://doi.org/10.1038/srep05408.
38. Norac Concepts Inc. 2008. Thiram 75 WP wettbale powder fungicide. Norac Concepts Inc., Guelph, Ontario, Canada.
39. Darsonval A, Darrasse A, Durand K, Bureau C, Cesbron S, Jacques M-A. 2009. Adhesion and fitness in the bean phyllosphere and transmission to seed of *Xanthomonas fuscans* subsp. *fuscans*. Mol Plant Microbe Interact 22:747–757. https://doi.org/10.1094/MPMI-22-6-0747.
40. Mohle-Boetani JC, Farrar JA, Werner SB, Minassian D, Bryant R, Abbott SLS, Vugia DJ. 2001. *Escherichia coli* O157 and *Salmonella* infections associated with sprouts in California, 1996-1998. Ann Intern Med 135:239–247. https://doi.org/10.7326/0003-4819-135-4-200108210-00008.
41. Mahon BE, Ponka A, Hall WN, Komatsu K, Dietrich SE, Siitonen A, Cage G, Hayes PS, Lambert-Fair MA, Bean NH, Griffin PM, Slutsker L. 1997. An international outbreak of *Salmonella* infections caused by alfalfa sprouts grown from contaminated seeds. J Infect Dis 175:876–882. https://doi.org/10.1086/513985.
42. Guo X, Chen J, Beuchat LR, Brackett RE. 2000. PCR detection of *Salmonella enterica* serotype Montevideo in and on raw tomatoes using primers derived from *hilA*. Appl Environ Microbiol 66:5248–5252. https://doi.org/10.1128/AEM.66.12.5248-5252.2000.
43. Zansky S, Wallace B, Schoonmaker-Bopp D, Smith P, Ramsey F, Painter J, Gupta A, Kalluri P, Noviello S. 2002. From the Centers for Disease Control and Prevention. Outbreak of multi-drug resistant *Salmonella* Newport—United States, January-April 2002. JAMA 288:951–953.